GUIDELINES ON THE BEST PRACTICES TO ASSESS THE IMPACT OF CHEMICAL POLLUTION ON CETACEANS / TO MEASURE THE CHEMICAL CONTAMINATION ON CETACEANS

- DRAFT REPORT -

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This report has been prepared by M. Cristina Fossi and Cristina Panti of the University of Siena. *The version of this report presented to the Eighth Meeting of the Parties to ACCOBAMS (MOP8) is not yet reviewed by the Scientific Committee of ACCOBAMS. This review will be organized after MOP8 and the final version of the report will be published on the ACCOBAMS website as soon as it is available.*

1. Standardization of protocols for samples collection and analysis on chemicals contaminants

The methodology of this tool follows the methods described in the literature based on the work of responsible bodies such as the IWC and ACCOBAMS/ASCOBANS for monitoring chemicals contaminants and micro and macro litter ingested by marine mammals including recent advances in the field within the framework of several projects focused on this issue as the Interreg Med Plastic Busters MPAs project to address the recent.

The presence of chemicals contaminants and the ingestion of macrolitter and microlitter by marine mammals such as deep diver cetacean species (*Physeter macrocephalus, Ziphius cavirostris),* coastal and pelagic odontocetes (*Tursiops truncatus, Stenella coeruleaolba, Delphinus delphis, Grampus griseus, Globicephala melas*), mysticete (*Balaenoptera physalus*), and the potential related effects can be investigated and/or monitored in:

- **Dead organisms** which may have been stranded ashore, found at sea, etc.
- **Free ranging organisms** that have been sampled at sea by remote dart sampling.

The marine mammals are protected species, therefore only authorized people can handle live and dead animals or parts of them. Upon finding the animal, its management and recovery should be reported and coordinated with the responsible Authorities. Permits released by national competent authorities are required for cetacean biopsy sampling. Note that a CITES permit is asked if a specimen or sample has to be sent/received.

A common protocol for samples collection and preparation for toxicological analyses need to be established.

1.1 Toxicology investigation in stranded cetaceans

In theory, all stranded cetaceans in a good state of conservation can be used to some extent in ecotoxicologic investigation (Godard and Fossi 2018). Skin as well as blood and all internal organs and tissues can be suitable materials for a wide range of analyses. Different sample matrices may be more suited to particular biomarker or residue analyses than others, depending on the contaminant or class of chemicals of interest, the time between death and sampling, and the sample storage condition. Geraci and Lounsbury (1993) and Mazzariol et al. (2011) thoroughly covered specimen and data collection in the case of marine mammal strandings. Whenever possible and before postmortem examinations, the stranded cetaceans need to be measured for total length and weighed. When weight measurement is not possible, the total length may be used to estimate weight according to formulas specific to the species investigated. Ideally, sample collection for all organs and tissues needs to be performed by necropsy-authorized personnel for later microscopic examinations (i.e., histopathology, immunohistochemistry, and ultrastructural investigations), as well as virologic and microbiologic investigations. Teeth and baleens can also be sampled for age determination (two teeth for each animal in odontocetes), while gastric contents can be collected during opening of the stomach complexes for the examination of organic, inorganic (including marine litter), and parasitic loads. Skin samples can be collected for genetic, stable isotope, fatty acid, hormone, biomarker, and chemical analyses, as well as cell and organotypic cultures. Most analyses are suitable with frozen storage conditions, but some require storage at various temperature or in various media, buffers, or solutions.

Relevant information on this section derived from the Joint ACCOBAMS and ASCOBANS document "**Best practice on cetacean postmortem investigation and tissue sampling**" (Lonneke L. IJsseldijk, Andrew C. Brownlow, Sandro Mazzariol, 2019).

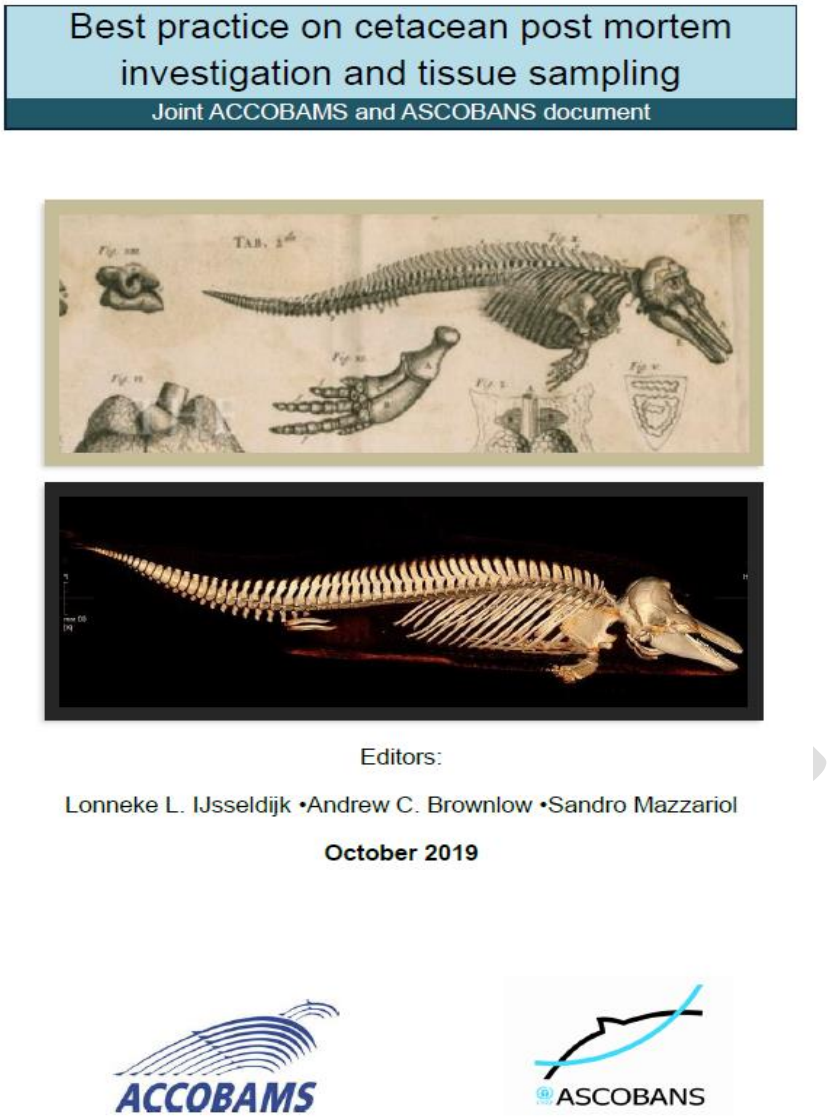


Figure 1. Joint ACCOBAMS and ASCOBANS document "Best practice on cetacean post mortem investigation and tissue sampling" (Lonneke L. IJsseldijk, Andrew C. Brownlow, Sandro Mazzariol, 2019).

Monitoring dead stranded cetaceans offers an often unique opportunity to gain insights into the health of, and threats and stressors affecting, marine ecosystems (Gulland & Hall 2009; Van Bressem et al. 2009). Information derived from the systematic examination of stranded carcases can provide insights into the at-sea population not easily acquired through other means, indeed strandings data is the major source of information available for some species (Reyes et al. 1991; Pyenson 2011).

Conservation status or decomposition level

When approaching to a dead organism, the conservation status is important for the diagnosis of the cause of death and for the analysis to be carried out on it.

With regards to the status of the dead cetaceans, 5 different situations can be observed:

Figure 2. Condition status and codes (**DCC**) for stranded organisms.

- **Level 1- Extremely fresh carcass, just dead**: the animal is found live or deceased at most by 2 h, adequate for *litter ingestion investigation, chemical analysis and biomarkers analyses.*
- **Level 2** - **Fresh carcass** (Death within 24 h): normal appearance with minimal damage from scavenger animals; normal smell; minimal skin dehydration and rippling of the skin, and apparent mucous membranes; clean and shiny eyes; uninflated carcass, tongue and penis not protruding. *Adequate for litter ingestion and chemical analyses.*
- **Level 3 - Moderate decomposition.**: Whole carcass, with evident swelling (tongue and penis protruding); skin not integrated with detachment areas; possible damage from scavenger animals; slight characteristic smell; apparent dry mucous membranes; eyes introflexed or missing. *Adequate for litter ingestion and chemical analyses.*
- **Level 4 - Advanced decomposition**: The carcass may be intact, but collapsed; wide areas of skin disepithelialization; severe damage from opportunistic animals; strong smell; muscles and blubbers easily removable and detachable from the bone; liquefaction of internal organs; allows to measure biometric data and assess the *presence/absence of ingested plastic and chemical analyses*.
- **Level 5- Mummified or skeletal remains:** Often with dehydrated skin and dry over the bones; completely dry; the analyses of litter ingestion or chemicals are not possible.

Below is reported the table **(Table. 1)** where the conservation level of the specimens is related to the different types of applicable investigations, including contaminant investigations and identification of the Marine Litter ingested.

Table 1. Recommendation for tissue sampling considering carcass **DCC**. Shading: green ***V*** indicates the process is of potential use in carcasses of the indicated DCC; grey (*V*)indicates that there may be limitations and red ***V*** indicates the procedure is not recommended/very unreliable, due to post mortem autolysis (From Best practice on cetacean post mortem investigation and tissue sampling" (Lonneke L. IJsseldijk, Andrew C. Brownlow, Sandro Mazzariol, 2019).

Analytical procedure	DCC 1	DCC 2	DCC 3	DCC 4	DCC 5	Comments/recommendations
Genetics	✓	✓	✓	✓	✓	For DCC4 or 5: paleopathological procedures may be required on account of degraded DNA (eg extracting DNA from bone medulla)
Diet and marine debris	✓	✓	✓	✓	(✓)	If GIT is not intact, eg from post mortem scavenger damage, results are compromised
Age determination	✓	✓	✓	✓	(✓)	
Fatty acids and stable isotopes	✓	✓	✓	✓	(✓)	Depending on analysis planned
Parasitology	✓	✓	✓	✓	(✓)	Depending on analysis planned
Morphometrics	✓	✓	✓	(✓)	(✓)	Girth measurements can be disrupted by bloating due to autolysis in DCC4-5
Gross pathology	✓	✓	✓	(✓)	(✓)	Recommended for DCC4-5 in cases of forensic investigation
Reproductive studies	✓	✓	✓	(✓)	✘	
Toxicology	✓	✓	✓	(✓)	✘	Depending on pollutants. DCC1-2 for biomarker investigation.
Ear investigation	✓	✓	✓	✘	✘	Inner ear analysis specifically: DCC1, histopathology of fixed ears possible up to DCC3
Microbiology	✓	✓	(✓)	(✓)	✘	Depending on analysis planned. For DCC3-4 microbiology can still be worthwhile for detection of certain bacteria and fungi using specific culture methods. Should a septicaemia be suspected in DCC3-4 animals, then microbiological investigations should be undertaken on the kidney, as this is resilient to microbial post mortem invasion using specific culture methods.
Histopathology	✓	✓	(✓)	(✓)	✘	Recommended for DCC4-5 in cases of forensic investigation
Virology	✓	✓	(✓)	✘	✘	Depending on analyses planned.

Biotoxins	✓	✓	(✓)	✗	✗	
Gas bubble analysis	✓	✓	✗	✗	✗	If this procedure is conducted: it should be done first, before undertaking further assessments and dissections, particularly prior opening any part of the vascular system or removing the head.
Serology	✓	(✓)	(✓)	✗	✗	Advisable both on blood serum and on cerebro-spinal fluid, the latter of which should be collected as soon as possible. In heavily autolyzed specimens, alternatives are "juice" obtained from skeletal muscle or lung, vitreous humour or pericardial fluid
Clinical chemistry	✓	✗	✗	✗	✗	Vitreous humour is a possible option in decomposed cases . Care is needed however to ensure sufficient baseline data are available for the analyte in the species under investigation.

1.1.1. Samples Collection

The choice of sampling for toxicological assessment should be made from the Stranding Task Force, operating in the ACCOBAMS area, in collaboration with the laboratory undertaking the processing of the samples.

Discovery circumstances – including entanglement and bycatch

Note the **circumstances** among the 5 categories:

- *Stranding**: Animal found stranded on the beach or in the shoreline,
- *By-catch**/*Fisheries*: Animal accidentally captured by fishers (e.g. ingestion of a hook, trapped in a net, brought back by fishers, etc.) during fishing operations.
- *Found at sea*: Animal discovered on sea surface.

** If possible, the type of interaction with human activities and impact observed or suspected on dead or live stranded individuals should be deduced from external or organs observations during the necropsy and complemented with veterinarian examinations. Also, an inspection of the oral cavity should be conducted for the presence of foreign material.*

Biometric Measurements and sex determination

Several basic and optional body lengths can be measured (in centimetres, precision 0.01 cm), as well as the weight.

The sex (male or female) should be noted, which can be determined by observation of sexual characters. Otherwise, specify by NI (for Not Identified).

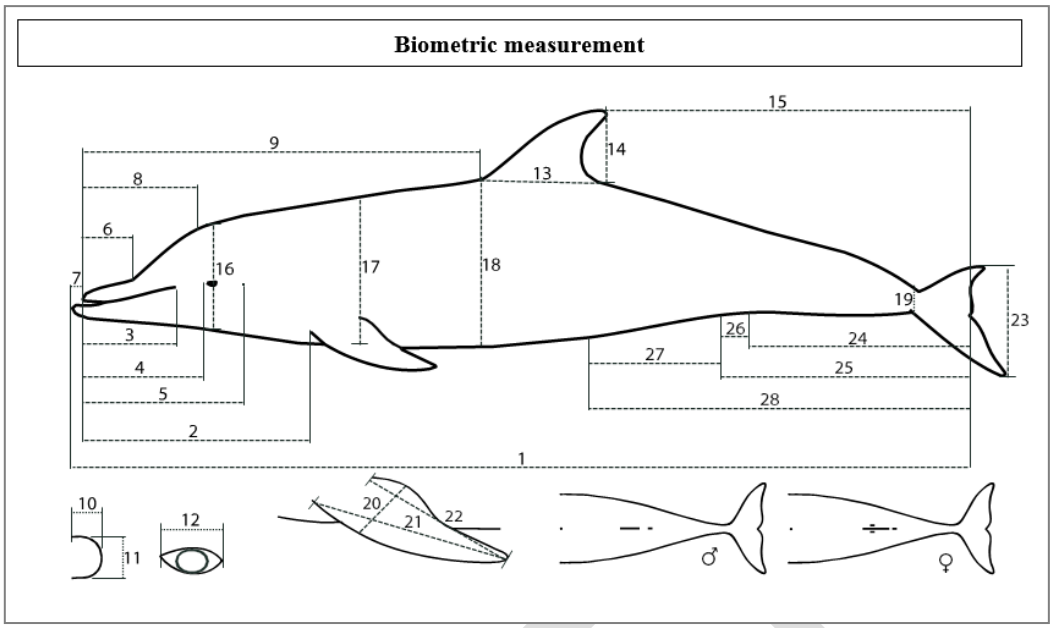


Figure 3. *Cetacean biometric measurement (from http://mammiferimarini.unipv.it/).*

Necroscopy and Health status

Necroscopy should be performed under the authorization needed at National level and with the presence of a veterinarian.

The body condition of a cetacean can be assessed by looking along the dorsal axis of the animal (poor, fair, good). The dorsal muscle mass (epaxial muscle) to either side of the dorsal fin of a robust animal will be rounded or convex. A thin animal will have a slight loss in epaxial muscle girth and could have a minor sunken aspect to the dorsal-lateral body. An emaciated animal will have a greater loss of epaxial muscle girth and will be concave down the dorsal-lateral body. Emaciated animal may also have more prominent indentation at the nape.

In addition visual inspection of the animal's fat reserves at the dorsal fin is recommended. Choose among the 3 categories:

- Thin;
- Fat;
- Normal.
- Not recorded (NR)

Extraction of the gastrointestinal system:

- Expose the gastrointestinal system (GI) by removing all excess attached tissues, the heart and liver of the animal. Clamp the oesophagus proximal to the mouth and clamp the colon, the closest to the anal orifice.
- Remove the entire GI and place it on the examination surface or isolate the different portions of GI (oesophagus, stomach, intestines) by strangling and cutting between 2 clamps the gastro-oesophageal sphincter and the pyloric sphincter. This operation is easier if done by at least 2 operators.
- During the whole procedure, airborne contamination should be prevented as much as possible.

Gut content analysis and marine litter isolation

- Before opening up the digestive tube, examine the outer wall to observe possible perforations by foreign bodies or areas of necrosis. Also, note any eventual secondary lesions, particularly a peritonitis following on a perforation of the digestive tube, an invagination of the digestive tube, an occlusion, etc. Photograph every lesion observed, taking care to get an overall view as well as close-up (macro-lens) photographs. Pictures must be stored referring to the code corresponding to the animal examined, describing the lesion in the description of the subject.
- The three parts of the gastrointestinal system (i.e. oesophagus, stomach, intestines) should be removed by adding a second strangling at the cut edge to prevent spillage of the contents. Each GI section should be opened lengthways using a scissor and slide the material directly out of the section onto a 1mm mesh sieve. The content should be cleaned with abundant tap water to remove the liquid portion, the mucus and the digested unidentifiable matter. Content should be inspected for the presence of any tar, oil, or particularly fragile material, and should be subsequently removed and treated separately. It should be then reported in the column “Notes” of the sampling sheet. All the material should be rinsed, collected in the 1mm sieve, and should be placed in tubes or in zipped bags, reporting the sample code (individual code, respective GI section) and stored at -20 °C, pending the laboratory analyses.
- NOTE: At this stage, for the optional differentiation of litter and microlitter, the material should be slid out of the section directly onto a 5mm mesh sieve superposed on a 1mm mesh sieve. Then, proceed with the rinsing and the storing of the material collected as described above, for both 1- and 5-mm sieves, reporting the samples code (individual code, respective GI section and size class (>5mm or 1-5mm)).
- If possible, follow the protocol developed in Corazzola et al (2021), which allows the simultaneous multidisciplinary analysis of GI by the implementation and standardization of a new methodological approach to the GIT of marine mammals. This protocol allows the collection of samples for different disciplines at the same time, performing the respective analyses, interpret and compare their results in a multidisciplinary way. The compatibility of multiple analyses allows the gaining of more information about the cause of death of stranded marine mammals and to enhance the knowledge of their biology and ecology.

The limit detection for MSFD is 1 mm. Building on the findings of the testing phase of the Plastic Busters MPAs project, it isrecommended to also examine the fraction 0.1-1 mm.

Figure 4. A new prototype to isolate macro and microplastics in the gastrointestinal tract of stranded cetaceans (Corazzola et al., 2021).

Tissue collection

Before sampling the contents of the GI for the subsequent contaminant analysis, collect about 10g of each of the following tissues (level 1-4), wrap them in aluminium paper and store at -20 ° C:

- Muscle
- Liver
- Blubber (include skin) fat taken at the base of dorsal fin
- Kidney
- Brain (if possible include cerebrum and cerebellum)

In case of **Level 1 specimen** (max 1-2h after death):

- Blubber (include skin) for analysis of biomarkers analysis and contaminant analysis: take 10-20g from preserved in aluminum paper,store in liquid nitrogen or dry ice, and then place at -80 °C.
- Liver for biomarkers analysis and contaminant analysis: 10g in aluminum paper, store in liquid nitrogen or dry ice, and then place at -80 °C.Blood for contaminant analysis: 5-10 ml in tubes and store store in liquid nitrogen or dry ice, and then place at -80 °C.Each tissue stored in aluminium foil or Eppendorf must be labelled with the standard identification code of the animal.

Additional Analytical Information

- It is recommended to archive duplicate samples of blubber, muscle, liver, kidneys and brain for subsequent persistent organic pollutants (POPs), plastic additives, trace elements and/or fatty acids analysis. Due to the potential for traces of contaminants in the sample to adsorb or absorb onto plastic, and vice versa, tissues destined for POP analysis should only come into contact with stainless steel, aluminium, glass or Teflon. Samples are most conveniently wrapped in standard catering-grade aluminium foil (shiny side out; do not use recycled foil as this might contain plastic particles) before being stored in standard plastic containers
- Milk samples should be collected from any lactating females and stored in a glass container prior to POP analysis. If the container has a plastic cap, it is recommended to cover the opening with aluminium foil (shiny side out) to prevent the sample from contacting the (plastic) cap for the reasons outlined above.
- Samples of brain, muscle, liver and kidney can be sampled for trace element analysis. These samples should not come into contact with any metals other than stainless steel and thus can be stored in plastic containers. Bone and/or blood samples are also suitable for heavy metal analysis.
- If a foetus is present but too small for full post mortem examination, the whole foetus and (parts of) its placenta can be wrapped in aluminium foil (shiny side out) for POP analysis. For foetuses that can be dissected, if is recommended to conduct a full post mortem investigation with individual tissue sampling.
- Storage: The minimal size of samples for trace elements and organochlorine analysis is 10g of solid tissue or 10 ml for milk. Samples should be stored frozen at -20°C until analysis. For DCC1-2, samples should be stored at -80°C in liquid nitrogen or RNA later for Real-Time (RT) PCR for Biomarkers investigations.

1.2. Toxicology investigation in free-Ranging Cetaceans

Several international institutions, such as the International Whaling Commission (IWC) have encouraged research on panels of sensitive nonlethal biomarkers, combined with analyses of persistent, bioaccumulative, and toxic (PBT) residues in skin biopsies of free-ranging animals. This approach can help define the health status of cetacean species with respect to multiple threats and supports IWC projects such as Pollution 2000+ and Pollution 2020 and Pollution 2025. From an ecotoxicological perspective, it is preferable to obtain samples from live free-ranging animals with collection methodologies that pose no or minimum disturbance. The most useful samples for non-destructive studies in cetaceans are skin biopsy specimens, obtained remotely by dart. The biopsy dart method has been used successfully on a range of cetacean species worldwide and is considered relatively benign (Noren and Mocklin, 2012). The response of cetaceans to skin biopsy collection is considered low in odontocetes and low to moderate in mysticetes, while wound healing appears to be rapid, with no apparent adverse health effects (Noren and Mocklin, 2012). Cetacean skin biopsies are suitable for hazard assessment of free-ranging cetaceans (Fossi et al., 1992, 2013, 2014, 2016, 2018; Godard et al., 2004; Godard- Codding et al., 2011). Various dart methodologies have been used successfully, as reviewed in Noren and Mocklin (2012). Skin biopsies (epidermis and dermis/ blubber) from free-ranging dolphins (such as *Tursiops truncatus, Stenella coeruleoalba*) can be obtained using an aluminium pole armed with biopsy tips (e.g., 0.7 cm ø, 3.0 cm length) or with a crossbow and darts. Skin biopsies from large odontocete (*Physeter macrocephalus*) or mysticete (such as *Balaenoptera physalus* or other baleen whale) species can be obtained with a crossbow or air gun and darts armed with tips (e.g., 0.9 cm ø, 4.0 cm length). Several models of crossbows (such as a Barnett Wildcat II crossbow with a 150-pound test bow), air guns, and darts (preferably untethered and with or without prongs of different angles in the tip) are available. To avoid the possibility of infection, the bolt tip needs to be sterilized before deployment. Biopsy samples can be taken between the dorsal fin and the upper part of the caudal peduncle upon approaching the animal at a suitable distance and speed as specifically permitted for the species and research project. The skin biopsy needs to be stored immediately in the proper conditions required for intended analyses. Common storage conditions include frozen, as is, in liquid nitrogen, dry ice, or −80 and −20 C freezers or stored either cold or at room temperature in cell medium, buffer, or specific reagents. Skin biopsy is a powerful tool for ecotoxicological studies for the following reasons: (1) it allows collection of a large number of samples across a wide geographic range; (2) it allows collection of sequential samples from the same animal if identified by photo identification or genetics; (3) it is suitable for residue analysis of many contaminants including dioxin-group chemicals (suitable for calculation of TEQs), other halogenated aromatic hydrocarbons, polycyclic aromatic hydrocarbons (PAHs), plastic additives (phthalates), and heavy metals; (4) it is suitable for several biomarker analyses (see the next section) and cell and organ culture. A number of successful studies show that cetacean skin biopsies are a powerful nonlethal tool for assessing ecotoxicological risk in marine mammals and aspects of feeding ecology and food preferences.

Cetaceans: Remote dart biopsy sampling procedure

Biopsy samples can be taken between the dorsal fin and the upper part of the caudal peduncle upon approaching the animal at a suitable distance and speed as specifically permitted for the species and research project. The skin biopsy needs to be stored immediately in the proper conditions required for intended analyses. Common storage conditions include frozen, as is, in liquid nitrogen, dry ice, and at −80°C and −20° C freezers for long-term storage or stored either cold or at room temperature in cell medium, buffer, or specific reagents (e.g. RNA later). Skin biopsy is a powerful tool for ecotoxicologic studies for the following reasons: (1) it allows collection of a large number of samples across a wide geographic range; (2) it allows collection of sequential samples from the same animal if identified by photo identification or genetics; (3) it is suitable for residue analysis of many contaminants s; (4) it is suitable for several biomarker analyses and cell and organotypic cultures.

Sampling procedure

- Skin biopsies (epidermis and dermis/blubber) from free-ranging dolphins (such as *Tursiops truncatus, Stenella coeruleoalba*) can be obtained using an aluminium pole armed with biopsy tips (e.g. 0.7 cm ø, 3.0 cm length) or with a crossbow and darts.
- Skin biopsies from large odontocete (*Physeter macrocephalus*) or mysticete species (such as *Balaenoptera physalus* or other baleen whales) can be obtained with a crossbow and darts armed with tips (e.g. 0.9 cm ø, 4.0 cm length).

Figure 5. Skin biopsy collection close to the dorsal fin of a fin whale and a striped dolphin. Remote sampling of integument biopsies from mysticete can be obtained using a crossbow and darts armed with tips (0.9 cm ø, 4.0 cm length) (Godard and Fossi 2018).

Skin biopsy collection

Once the biopsy has been collected from the animal, it should be processed as soon as possible.

1. Unscrew the tip from the arrow using gloves, put the biopsy in a small bag and write on the bag the code of the animal (put it in the fridge or keep as cold as possible). If there is more than one animal to collect at the same time and you cannot process the biopsy immediately, use a refrigerated bag until the processing.

Figure 6. *Arrow and tip (left) and tip on the aluminum pole (right) with the collected biopsy.*

2. Remove the biopsy from the tip using tweezers, paying attention to keep the biopsy entire and put the biopsy on a clean petri dish.

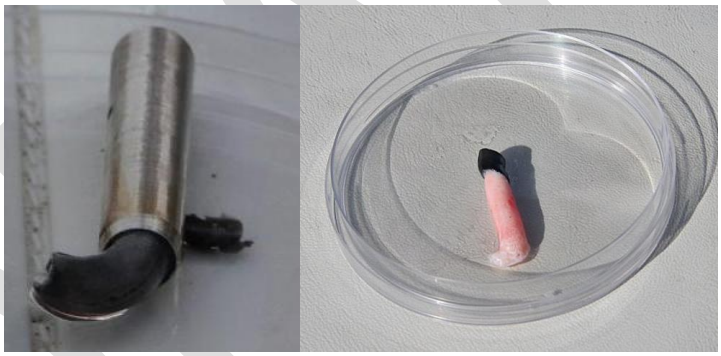


Figure 7. *Biopsy sampled: left) biopsy tip, right) biopsy on a Petri dish.*

3. With a clean scalpel cut at least two pieces of skin (about 0.2x0.2 cm each) from the top of the biopsy (yellow squares) and put the separated aliquots of skin in two 0.5 ml Eppendorf. Whenever possible, for larger biopsy, divide the sampled biopsy in 4-5 different aliquots. Wrap up the skin+blubber (red square) in a small aluminium foil and put the biopsy in a 2 ml Eppendorf (contaminants and protein analysis). Organotypic slice cultures should be potentially performed in specialized laboratory.
Write with a marker the code of the animal on the Eppendorf tubes, and, if possible, put inside the 2 ml tube a small piece of paper with the code of the biopsy written with the pencil, in order to be sure to not lose the name of the sample. During the operation, fill in the sampling sheet.

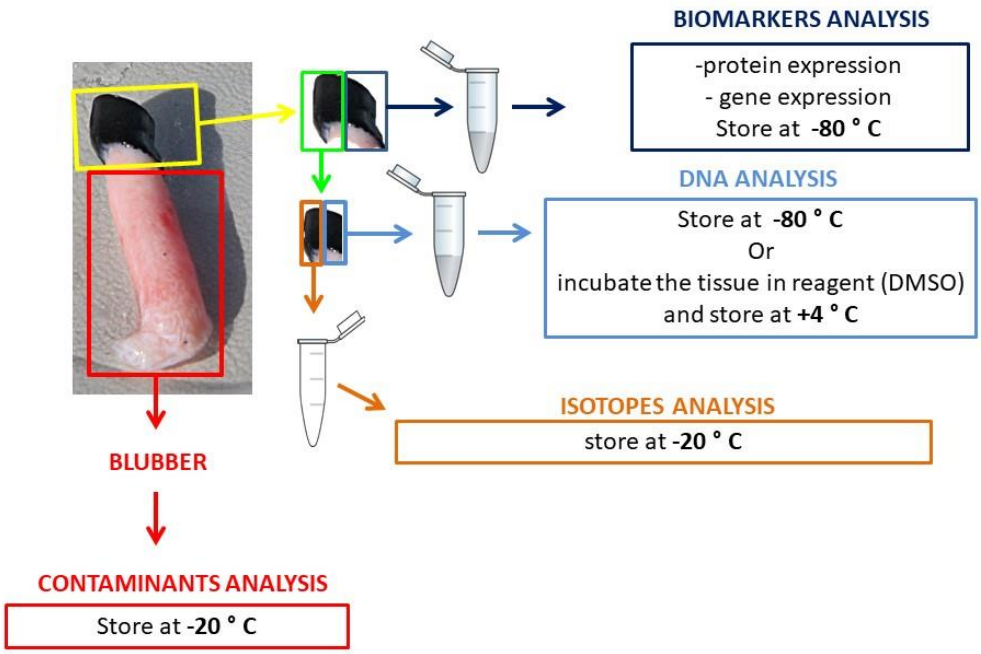


Figure 8. Biopsy with blubber (red square) and dermal part (yellow squares) and relative storage conditions for each aliquot.

4. Place the tubes in liquid nitrogen. The samples stored in RNAlater can be kept at room temperature for 24 hours and then stored at +4°C or -20°C for long-term storage.
5. Clean accurately the tips and boil them in freshwater for ten minutes to avoid cross contamination and pathogen transmission among individuals. If boiling the tips is not possible, rinse them with ethanol. Rinse with ethanol also the scalpel and the tweezers.

Storage conditions

For skin biopsy:

- ***Dermal tissues (skin):***
  - 40-60 mg in cryo-vial frozen at -80 °C (protein expression analysis/-omics analysis)
  - 30-50 mg in RNAlater at -20 °C or in cryo-vial frozen directly at -80 °C (gene expression analysis/transcriptomics)
  - 20-30 mg in 20% saturated DMSO with NaCl or in cryo-vial frozen directly at -80 °C (sex determination and genetic analysis)
  - 20-30 mg in cryo-vial frozen directly at -80 °C (stable isotopes analysis)
- ***Blubber tissues (fat):***
  - entire blubber in aluminium foil directly at -80 °C (contaminants analysis)

Faeces collection

- For **free-ranging cetaceans**, faeces collection can be occasional and discontinuous, and generally, available only for fin whale. In case of localization of faeces, they should be collected as much as possible with a net (mesh size 200 µm or less) and put in falcon tubes for subsequent analysis: liquid nitrogen for contaminants and biomarker analysis, -20 °C for litter analysis.

1.3. A threefold monitoring approach to detect marine litter ingestion, contaminants, and toxicological impact in cetaceans

Given the multiple exposure to anthropogenic stress, such as marine litter and contaminants and on marine organisms should be assessed using a threefold approach. The application of the threefold approach can elucidate not only the rate of ingestion in cetaceans, but also the multiple sublethal stresses in the short and long term. Each of the three investigation tools that make up the threefold approach can be applied independently or simultaneously using different methods according to the species and whether the animal is stranded or free ranging.

After the sampling phases described above (both in stranded and free-ranging animals), the analytical phases can be proceeded.

Each of the three investigation tools that make up the threefold approach can be applied independently or simultaneously using different methods according to the species and whether the animal is stranded or free-ranging.

The threefold approach comprises the following elements:

- **Analysis of gastrointestinal content**: For stranded cetaceans, it is possible to detect the occurrence and rate of marine litter ingestion and any associated pathology through analysis of the gastrointestinal content, with a particular focus on plastics and microplastics.
- **Analysis of the levels of POPs, plastic additives, and emerging contaminants**: An indirect approach can be used for free-ranging as well as stranded animals. The levels of chemical compounds can be measured to evaluate the exposure pollution .
- **Analysis of biological end-points**: Biomarker responses and omics analysis can be used to detect the potential toxicological effect related to exposure to multiple stressors, contaminants and plastic ingestion, in free-ranging individuals or in stranded organisms up to a few hours after death.

Litter analysis and classification

Macrolitter detection in stranded organisms

- Sort prey or litter items from the gastrointestinal tract into separate categories under a stereomicroscope, taking care of recording their weight.
- Measure the size of litter items and classify litter.

In addition, the following parameters should be recorded:

- For all categories (litter and other elements): the dry mass (grams, precision 0.01 g) of each category; dry the sample at room temperature during 24h minimum or in a stove at 35ºC for 12h.

- For litter categories only: the number of fragments and items in each category: a fragment is a piece of litter that can be identified, whilean item is a set of fragments that seem to originate from the same piece of litter
- For the plastic litter categories only the total number of plastic fragments per colour, shape and dimension category.
- Analyse at least 10% of the detected plastic by FTIR (Fourier Transform Infrared Spectroscopy) or Raman spectroscopy to determine the polymer composition and confirm the polymer origin of the detected particles.

Microlitter detection

- Examine the filter in the Petri dish under a stereomicroscope for particles resembling microplastics. Cover the filter with glass lids during observation to avoid the contamination of the sample.
- Photograph, count and record the type, colour and maximum length of microplastic particles using image analysis software and categorize microplastic particles.
- Analyse at least 10% of the detected microplastics by FTIR (Fourier Transform Infrared Spectroscopy) or Raman spectroscopy to determine the polymer composition and confirm the polymer origin of the detected particles.

Microlitter detection in faeces

For free-ranging faeces, samples should be dried and digested using KOH 10%, then the solution filtered and litter should be classified. The dry mass (grams, precision 0.01 g) of each category should be recorded after drying at room temperature for at least 24h or at 35 °C for 12 h.

Litter categories

Categorize marine litter according to the categories showed in Table 2. The categorization of the gastrointestinal tract contents and excreta is based on the general “morphs” of plastics (sheet-like, thread-like, foamed, fragment, other) or other general rubbish or litter characteristics. This is because in most cases, particles can’t be unambiguously linked to particular objects. But where is possible, under notes in datasheets, the items should be described and assigned a litter category number using the “Joint List” developed by the TSG ML group (Fleet et al., 2021). In addition, it is important to measure and quantify also natural items (food and/or no food).

Table 2. *Classification of marine litter items plus food remain and natural no food remain (from INDICIT 2018).*

BIOTA categories for contents of digestive tract			
PLA	**PLASTIC**	**acronym**	all plastic or synthetic items: note number of particles and dry mass for each category
IND	**pellets**	**ind**	industrial plastic granules (usually cylindrical but also oval spherical or cubical shapes exist)
	probab ind?	**pind**	suspected industrial, used for tiny spheres (glassy, milky, ...) (= microbeads)
USE	**sheet**	**she**	remains of sheet, eg from bags, cling-foil, agricultural sheets, rubbish bags etc
	thread	**thr**	threadlike materials, eg pieces of nylon wire, net-fragments, woven clothing; includes 'balls' of compacted material
	foam	**foam**	all foamed plastics, polystyrene foam, foamed soft rubber (as in matrass filling), PUR used in construction etc
	fragments	**frag**	fragments, broken pieces of thicker type plastics, can be bit flexible, but not like sheetlike materials
	other	**Poth**	any other, incl elastics, dense rubber, cigarette-filters, balloon-pieces, softairgun bullets, objects etc. DESCRIBE!!
RUB	**OTHER RUBBISH**	**acronym**	any other nonsynthetic consumer wastes: note number of particles and (in principle) dry mass for each category
RUB	**paper**	**pap**	newspaper, packaging, cardboard, includes multilayered material (eg Tetrapack pieces) and aluminium foil
	kitchenfood	**kit**	human food remains (galley wastes) like onion, beans, chickenbones, bacon, seeds of tomatoes, grapes, peppers, melon etc
	other rubbish	**rubvar**	other various rubbish, like processed wood, pieces of metal, metal air-gun bullets; leadshot, paintchips. DESCRIBE
	FISHHOOK	**hook**	fishing hook remains (NOT FOR HOOKS ON WHICH LONGLINE VICTIMS WERE CAUGHT - THOSE UNDER NOTES)
POL	**POLLUTANTS (INDUS/CHEM WASTE)**	**acronym**	other non-synthetic industrial or shipping wastes (number of items and mass per category (wet for paraffin)
POL	**slag/coal**	**slag**	industrial oven slags (looks like non-natural pumice) or coal remains
	oil/tar	**tar**	lumps of oil or tar (also note as n=1 and g=0.0001g if other particles smeared with tar but cannot be sampled separately)
	paraf/chem	**chem**	lumps or soft mush of unclear paraffin, wax like substances (NOT stomach oil!); if needed estimate mass by subsampling
	featherlump	**confea**	lump of feathers from excessive preening of fouled feathers (n=1 with drymass) (NOT for few normal own feathers)
FOO	**NATURAL FOOD**	**foo**	various categories, depends on the species studied, and aims of study
NFO	**NATURAL NON FOOD**	**nfo**	anything natural, but which cannot be considered as normal nutritious FOOD for the individual

Collection of data

For each organism, an assessment is made of:

1. Frequency of occurrence (%) of ingested macro and microlitter for each species, calculated as the percentage of the individuals examined with ingested macro- and microplastics.
2. Abundance (N) of macro and microlitter ingested per individual (average number of items/individual) for each species, calculated as a total and per category. Since currently there are inconsistencies in the literature in reporting abundance of ingested litter, it is recommended to report average number of items per individual considering both all individuals examined and only individuals found with ingested macro and litter.
3. Total dry weight (g) of the detected waste expressed on grams (precision: second decimal place). This weight refers to each single category found in a specific organ (or faeces) of the specimen.

Other information as colour of items, polymer of the different items (at least 10% of the total items) and different incidence of litter in oesophagus, stomach and intestine, incidence and abundance are useful for research and impact analysis.

Analysis of cotaminants

Depending on the compounds and the tissue to be analysed, different methods should be applied to detect the presence of contaminants in cetaceans (**Annex I**).

The most common classes of contaminants detected in cetaceans are the following:

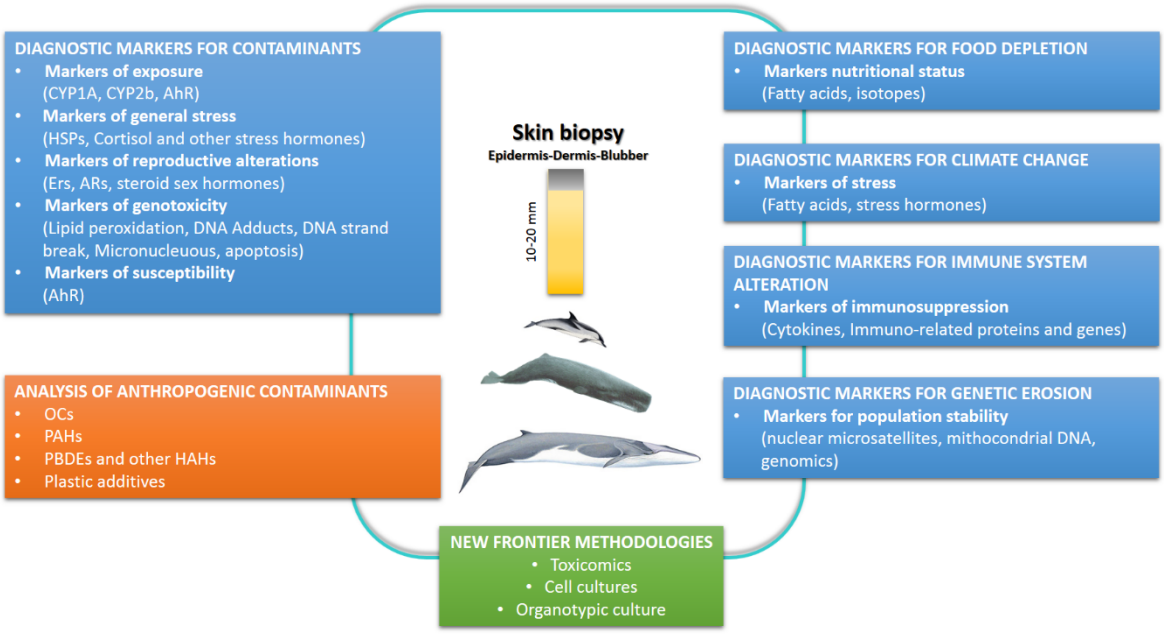
- *Organochlorine compounds (OCs):* This class of contaminants consider all those compounds characterized by the presence of chlorine, mostly of them are banned since several decades but still persist in the environment and biota. Among the compounds analyzed in the cetaceans from the ACCOBAMS there are: several congeners of PCBs, DDT and its metabolites, Hexachlorocyclohexane; exachlorobenze, polychlorinated diphenyl ether (PCDEs), polychlorinated terphenyls (PCTs), polychlorodibenzofurans (PCDFs), polychlorodibenzo-p-dioxins (PCDD) (Aznar-Alemany et al., 2021; Fossi et al., 2013).
- *Phthalates esters (PAEs):* a group of chemicals widely used as additives to make plastics more flexible and harder to break; they can interfere with endocrine system (Baini et al., 2018).
- *Bisphenol A (BPA)*: used in the production of polycarbonate, can have endocrine disrupting effects (Crain et al., 2007; Halden, 2010; Oehlmann et al., 2009) and the styrene and polyvinyl chloride monomer, used in the production of polystyrene and polyvinyl chloride (PVC), can be carcinogenic and/or mutagenic (Lithner et al., 2011; Papaleo et al., 2011; Xu et al., 2004).
- *Polybrominated diphenyl ethers (PBDEs):* they belong to the group of brominated flame retardants (BFRs), which are used in various polymeric materials such as plastic parts, resins, textiles, and other substrates to reduce their fire hazards (BSEF 2003; Król et al. 2012; Bartalini et al. 2019).
- *Per- and polyfluorinated alkyl substances (PFAS)*: are a large group of industrial chemicals (Jahnke and Berger, 2009) characterised by a linear or branched carbon chain (Ferrario et al., 2021) with an alkyl chain which is partly or fully fluorinated, typically containing between 4 and 18 carbon atoms (Jahnke and Berger, 2009). They tend to accumulate in cetaceans, few studies are available for the ACCOBAMS area (e.g., Sciancalepore et al., 2021)
- *Heavy metals and trace elements*: are well known environmental pollutants that accumulate in the bodies of cetaceans and potentially constitute a toxicological risk for the species. Numerous studies have been conducted over the last decades on trace metals in marine mammals (Riget and Dietz, 2000; Robin et al., 2012; Rojo-Nieto and Fernandez-Maldonado, 2017).

- Polycyclic aromatic hydrocarbons (PAHs): are a class of semi-volatile organic compounds with at least two fused benzene rings, which may have branches of aliphatic chains (alkyl-PAHs). Several PAHs, especially the High Molecular Weight compounds, are classified as toxic, some of which present mutagenic and carcinogenic properties. Studies on cetaceans inhabiting the ACCOBAMS area are available but scarce (Marsili et al., 2014; Marsili et al., 2001).

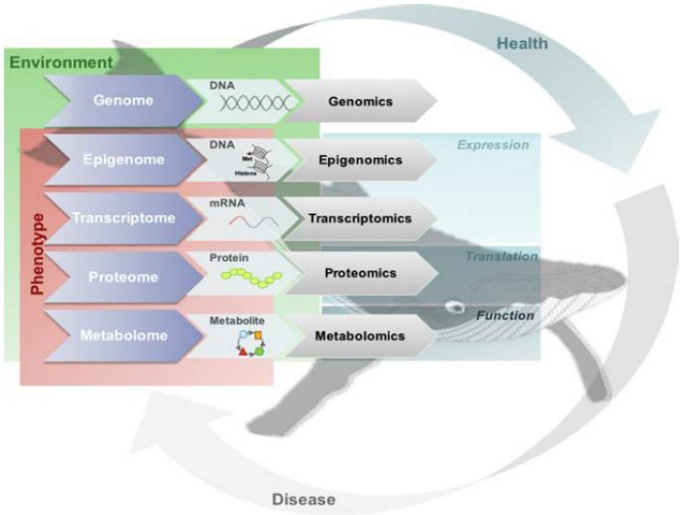
Biomarkers analysis

The toxicological effects associated with the presence of marine litter and related contaminants can be evaluated using a set of diagnostic and prognostic methodologies, by means of biomarkers.

Biomarkers have been selected on the basis of the level of biological responses to the exposure to contaminants. The selected biomarkers can diagnose different impacts related to: a) physical damages/effects of marine litter, b) exposure to/effect of chemicals, c) exposure to multiple stress (e.g., synergistic/antagonist effect of contaminants and climate change).



A



B

***Figure 9. A**) Skin biopsies as a diagnostic tools of chemicals exposures in marine mammals; **B**) Omics techniques in skin biopsies (from Mancia 2018).*

Starting from this initial list and building on the findings of the testing phase of the Plastic Busters MPAs project, the most suitable diagnostic tools to detect the presence and impact pollution onn marine mammals are proposed and reported in the the Plastic Busters MPAs final protocol.

Materials & Equipment for sampling

The following material and equipment are necessary for the correct application of the protocol (**stranded organisms**):

- Boots
- Camera
- Clamps (at least 6) or roast wire
- Clips with claws
- Containers for samples (Bottle/zipped bags)
- Cooler
- Cut-resistant gloves
- Garbage bag
- Glasses and protective mask or shield
- Nitrile Gloves
- Integral protective suit
- Measuring cylinders (2 L, 1L, 50cL; precision 0.1L)
- Measuring decimetre
- Measuring tape
- Metal containers
- Metal spoon
- Observation sheet
- Pen
- Permanent marker
- Precision balance
- Rope (to mark-off the zone)
- Sampling sheets
- Scalpel
- Scissors
- Sieve with 1 mm mesh
- Sieve with 5 mm mesh
- Transport bins or containers

The following material and equipment are necessary for the correct application of the protocol (**free-ranging organisms**):

- Aluminium foil
- Aluminium Pole
- Binoculars
- Camera
- Crossobow
- Cryoboxes
- Cryovials
- Darts
- DMSO (20% saturated with NaCl)
- Eppendorf (0.5 ml. 1.5 ml. 2.0 ml)
- Ethanol (70%, 100%)
- Falcon tubes
- Glass Petri dishes
- Gloves
- GPS
- Liquid nitrogen dewar (in alternate dry ice)

- Net (for faces collection)
- Paper and block-notes
- Paper towels
- Pasteurs
- Pencils
- Permanent markers
- Plastic Sealable bags
- RNAlater
- Ruler
- Scalpels
- Spare batteries
- Spare camera batteries and memories
- Thermic bags
- Tips (for crossbow and aluminium pole)
- Tweezers
- VHF Radio

Main reference documents

IPA-Adriatic NETCET, 2015. Standard protocol for post-mortem examination on cetaceans

Joint ACCOBAMS and ASCOBANS document "Best practice on cetacean post mortem investigation and tissue sampling" (Lonneke L. IJsseldijk, Andrew C. Brownlow, Sandro Mazzariol, 2019).

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ANNEX I

Table A-1. Tissues and methods to be used to detect Contaminants in marine mammals.

CHEMICAL COMPOUND	TISSUE/SAMPLE	ANALYTICAL METHOD
Phthalates	Blubber, muscle, liver, kidney, skin biopsy	Baini et al., (2017), Fossi et al., (2016), Savoca et al., (2018), Routti et al., (2021)
Bisphenol A	Muscle	Ballesteros-Gómez et al., (2009)
	Blubber, skin biopsy	Xue et al., (2016)
	Blood	Cobellis et al., (2009)
Polybrominated diphenyl ethers	Blubber, muscle, liver, blood, kidney, skin biopsy	Muñoz-Arnanz et al., (2016), (Zaccaroni et al., (2018), Bartalini et al., (2019), Baini et al., (2020), Aznar-Alemany et al., (2021)
Polycyclic aromatic hydrocarbons	Blubber, muscle, liver, blood, kidney, skin biopsy	Marsili et al., (2001)
Organochlorine contaminants	Blubber, muscle, liver, blood, kidney, skin biopsy	Marsili and Focardi, (1997), Bartalini et al., (2019), (Genov et al., 2019), Baini et al., (2020), Aznar-Alemany et al., (2021)
Per- and polyfluoroalkyl substances	Blubber, muscle, liver, blood, kidney, skin biopsy	Sciancalepore et al., 2021
Heavy metals	Blood, skin, kidney, skin biopsy	Correa et al., (2013)

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