EUROPEAN SCIENCE FOUNDATION
SETTING SCIENCE AGENDAS FOR EUROPE

Exploratory Workshop Scheme

Standing Committees for
- the European Medical Research Councils (EMRC)
- Life, Earth and Environmental Sciences (LESC)

ESF Exploratory Workshop on

Cryopreservation of ovarian tissue in cancer patients, farm animals and endangered species

Heidelberg, Germany, 15-17 May 2008

Convened by:
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Scientific report

1. Executive summary

Background

Fertility preservation in women with cancer and preservation of gametes of farm animals and endangered wildlife species are of increasing scientific and public interest. A solution to both topics may involve cryopreservation of ovarian tissue, followed by tissue re- or xenotransplantation or/and by production of mature oocytes in vitro. These techniques are highly complex and their efficacy is still very limited. Substantial progress in this field is most likely dependant on a successful and not yet performed interdisciplinary approach by different researchers from human reproductive medicine, veterinary medicine and biology.

Objectives of the workshop

The objectives of the workshop were:

- Integration of European clinicians and scientists and exchange of knowledge between the different specialties,
- Development of new approaches to successfully cryopreserve and re-transplant ovarian tissue,
- Preparation of manuscripts to share new approaches and interdisciplinary knowledge with other clinicians and scientists,
- Preparing the groundwork for future research co-operations.

Design of the workshop

The workshop was designed to allow an interdisciplinary discussion and finally integration of leading researchers in all different disciplines involved such as biology, veterinary medicine and medicine. Much attention was paid first to invite both, senior scientists, who are well integrated in the scientific community as well as outstanding junior scientists, performing substantial basic research and providing hands on experience. Second, to invite leading experts on human as well as of non-human tissues to integrate the knowledge of both fields.

With this concept, the workshop was able to search the not yet integrated fields of cryopreservation in humans, farm animals and endangered species in respect of basic and

clinical research, its relevance for and its impact on modern medicine, and its potential to support worldwide activities to protect endangered species and to preserve their gene pool.

Relevance of future activities addressed before and during the workshop

During the workshop it became obvious that progress in this field will essentially depend on the integration of all fields involved for the following reasons:

1. In humans cryopreservation of ovarian tissue in cancer patients has proved to be successful as demonstrated by 5 live births following re-transplantation of ovarian tissue. Nevertheless the efficacy of the techniques needs to be improved, ethical concerns needs to be addressed, indications more clearly defined and the techniques needs to be introduced in all European countries and areas requiring intensive network logistics on a very high scientific and clinical level.

   These goals can only be achieved if biologists and veterinarians co-operate with medical doctors, using their animal and research models, allowing essential progress and clinical work according to Good Medical Practice (GMP).
2. In non-humans, research groups are focussing on single species to develop techniques to cryopreserve ovarian tissue in order to develop different strategies to support activities to protect endangered species. The workshop proved that first most of these activities are still limited and restricted to a small number of species due to the very slow progress in this field and second that experts on human and non-human species have each developed techniques in their own fields, which ha the capacity to support both areas substantially provided a successful integration can be implemented.
3. Cryopreservation of ovarian tissue and gametes in humans and non-humans has a worldwide dimension and has the potential to have a high impact on quality of life for many people and for the potential preservation of endangered species. It also became clear that this topic is also of great relevance in Europe and that European researchers are in the frontline of this research, as demonstrated by the fact that four out of five children born as a result of transplantation of frozen/thawed tissue is performed in Europe and that more than 2/3 of all women having had frozen/thawed tissue transplanted are European. The high number of patients requiring fertility preservation because they face a gonadotoxic treatment and the alarming number of endangered species in Europe demonstrate that this topic deserves much attention in a European context. As the expertise to tackle the scientific and logistic hurdles is centered in a few but well integrated places around Europe, the integration of European research has

the potential to further advance and place Europe as the leading region within these topics.

2. Scientific content of the event

Indication and logistics in Europe in ovarian tissue banking

Ovarian tissue banking in humans

In humans, fertility protection by cryopreservation and banking of ovarian tissue must be considered as an integrated patient-orientated concept. This involves tissue procurement, transportation to the tissue establishment, processing, cryopreservation, storage and its further use (e.g. transplantation). The entire procedure need to fulfil the requirements imposed by relevant legal directives to become operative. Of uttermost importance are investigations on tissue integrity, follicle viability and on the future potential of the tissue, especially regarding its suitability for transplantation.

For most patients the indication for fertility preservation is an underlying oncological or haematological disease. Therefore a histopathological investigation on a small piece taken from the fresh ovary during or after the biopsy is advisable. This will also provide information on the presence and density of primordial follicles, constituting the source for mature oocytes following transplantation. In case of tissue transportation prior to processing, follicular viability should be documented by using a xenotransplantation procedure. The same approach should be undertaken to proof the safety and suitability of the applied freeze/thaw protocols. For those patients where the primary treatment option is future transplantation of ovarian tissue, small parts of ovarian tissue from different sites should be frozen separately for later molecular genetic or immunohistochemical analysis of candidate genes and gene-products associated with the underlying disease. This information is important to detect a possible spreading of the disease to the ovary and to provide information that the initial disease will not be re-induced through the transplanted tissue.

Future perspectives:

State of the art collection of human ovarian tissue, its processing and cryopreservation is primarily performed in similar institutions. The very strict regulations, as outlined in the relevant directives (e.g. EU 2006/86/EC) and the highly sophisticated evaluation of the tissue samples needed prior to cryopreservation and re-transplantation require specialised public

funded institutions and cryo-banks. As patients often are too sick to travel long distances to be operated in such institutions, decentralized tissue procurement and centralized tissue processing appears to be an option to solve this problem. In countries such as Denmark and Germany (www.fertiprotekt.de), network structures have already been implemented which can be adopted by other countries to allow the introduction of fertility preservation programmes in hospitals that do not have local expertise and can essentially cover the entire European population

Ovarian tissue banking in non-humans

In non-humans preservation of male gametes and embryos is a well-known procedure, while the long-term storage of female gametes remains a challenge for almost all species.

The ovary contains thousands to millions of early-staged follicles that even may be recovered viable a few hours after the animal's death. Thus, cryopreservation of the ovarian cortex in which almost all primordial follicles are located, could be used to preserve gametes and be used to restore fertility in animals. During the last 100 years, many hundreds of species have been lost, and a third of the breeding animals are threatened with extinction. To preserve genetic diversity, notably for the conservation of endangered species and many old and seldom used subspecies of farm animals, it is essential to conserve female and male gametes. Today, biotechnologies such as artificial insemination and embryo transfer are used in breeding programs and are well developed. However, even using these advanced techniques, there are problems due to the limited number of individuals that serve as the source of gametes, so that the risk of inbreeding is high, even in relatively large populations. To preserve genetic diversity, it is necessary to create gene banks of male and female gametes and embryos, using a very large number of individual donors. Cryopreservation of ovarian tissue could present means for enlarging the gene pool. It could be used in auto- or xenografts, or for in vitro culture of early-stage follicles and subsequent oocyte in vitro maturation (IVM) and fertilisation (IVF).

Future perspectives:

Non-human, non-gonadal tissue from endangered species is already cryopreserved in some tissue banks. However, these initiatives preserve genetic material which can hardly be used to breed species after extinction. It is therefore of utmost importance to cryopreserve material that can be used to breed endangered species. Techniques to cryopreserve gonadal tissue and to further process this tissue as well as specialized cryobanks must therefore be developed in

an interdisciplinary context. Presently, no European biobank with the focus of preserving gametes and embryos from rare animal and endangered species does to our knowledge exist.

Cryobiology of ovarian tissue and freezing techniques

In each cell type and tissue, it is important to minimize the ice crystal formation by properly adjusted freezing and thawing rates and by choosing a combination of cryoprotectants which optimally permeates all the tissue components. It is always a balance between the toxicity of the cryoprotectant and ice crystal formation.

Ovarian tissue is cryobiologically challenging because it contains many cell types with different sizes, and also specific extracellular matrix components. The oocytes are located in the ovarian cortical tissue mostly in the confinement of primordial follicles surrounded by a single layer of flat granulosa cells, or in primary follicles where the surrounding granulosa cells are cuboidal. The follicles are located within the dense stromal tissues composed of middle size cell and extracellular matrix with collagen bundles. The oocytes at these developmental stages are much smaller, 35 µm in diameter, than mature oocytes, but they are still large cells when compared to other cells types.

A successful cryopreservation method in such tissue always represents a compromise. The concentrations of cryoprotectants and the freezing rates have to be experimentally optimized.

Both slow programmed and rapid freezing methods have been studied in human ovarian tissue. Slow programmed freezing with healthy infants born after freezing and thawing has become the golden standard. In slow freezing, ice crystal formation is induced outside the cellular compartment by using a super cooled instrument. In connection with vitrification, there is no crystal formation. The glass like state is obtained with high concentration of cryoprotectants and very fast cooling rates. To avoid their toxicity, combinations of several cryoprotectants are often used. Also the thawing has to be rapid. According to recent experience, vitrification can result in better survival of the stromal cells, which appears to be a problem in many slow protocols.

A challenge is still to organize the freezing and vitrification methods and storage of the tissue so that it fulfills the requirements of the EU Tissue Directive. It can be done, but each laboratory has to validate the used methods according to these requirements.

Perspectives for the future

Progress in freezing techniques such as slow freezing and vitrification allow cryopreservation of gonadal tissue in humans, in farm animals and some endangered species. However, the efficacy of these techniques is still limited. Furthermore, the biological diversity of gonadal tissue of different species requires development of species specific protocols and does not allow the use of one general protocol. The workshop clarified that different standardized freezing protocols need to be developed and evaluated with aim of being able to apply them to different species with ovaries of similar biological characteristics. Systematic approaches involving input from different cryo-specialists are required to achieve this goal in the future.

Cryopreservation and transplantation of human tissue

Every year an increasing number of women receive treatment involving cytotoxic chemoradiotherapy for various malignant and nonmalignant diseases. Women who face the risk of premature or imminent ovarian failure caused by cytotoxic therapy may preserve their fertility potential with ovarian tissue cryopreservation. Until recently, this technique could only be performed in few highly specialized institutions. However, with the latest advances in cryobiology, ovarian tissue cryopreservation is rapidly becoming a more widely offered technique by many IVF centers worldwide. The indications for ovarian tissue cryopreservation now extend beyond cancer. Even though the risk of reimplanting preexisting cancer cells is minimal or non-existent for most types of cancer, this risk needs to be ascertained according to the cancer type and disease stage. When the risk of ovarian metastasis is high, a heterotopic site is recommended for easy tissue monitoring. While orthotopic transplantation offers the possibility of natural conception, heterotopic transplantation maybe indicated if the pelvis is not suitable for transplant due to previous radiation or severe scar formation. Heterotopic transplantation requires aspiration of follicles and in vitro fertilisation.

Both approaches have resulted in pregnancies and live births. Around 20 patients have received transplantation with frozen/thawed ovarian tissue into either the ovaries or the pelvic side wall, or into heterotopic sites, resulting in the resumption of endocrine function up to 3 years and the birth of 5 healthy children.

Future perspectives

Despite the successful pregnancies several issues still need to be addressed. The best technique to cryopreserve and to re-transplant the tissue has not yet been determined. Endocrine treatments following re-transplantation to improve tissue survival must be developed and important topics such as the risk of spreading metastasis after re-transplantation need to be clarified. Since each patient diagnosed with malignant or other systemic diseases present with a unique clinical situation, a cased-based approach should be developed to define how much of the ovarian tissue is harvested as well as how much of the cryopreserved tissue is thawed to be transplanted. As the number of re-transplantations in each centre is limited, multicentre approaches will facilitate solution to these issues.

Cryopreservation and transplantation of non-human ovarian tissue

Cryopreservation and transplantation of whole ovaries

A major problem in ovarian tissue transplantation is follicular loss due to ischemic reperfusion injury. Studies suggest that many more follicles are lost during revascularization than by freezing and thawing procedures, and the key factor responsible for follicular survival is the postgrafting ischaemia, resulting in significant follicular loss and affecting long term functionality. A promising approach to reduce ischemic damage is cryopreservation of an intact ovary with its vascular pedicle. Application of freezing medium via vessels could enable faster penetration of the tissue by the medium as well as fast perfusion after re-transplantation studies with ovine ovaries have demonstrated that cryopreserved ovaries can be implanted successfully by microvascular anastomosis. The delivery of a lamb following transplantation has proved this technique to be feasible and promising in connection with ovarian tissue banking.

Cryopreservation of primordial follicles

Primordial follicles can be cryopreserved using two different techniques: (i) enclosed in ovarian tissue; or (ii) after isolation from the ovarian cortex. Most reported studies involve ovarian tissue cryopreservation and only a few papers describe freezing of isolated follicles. Isolated follicle cryopreservation has several potential advantages over ovarian tissue cryopreservation, but this technique is very challenging and still in its infancy. Nevertheless, cryo-preservation of follicles has proven successful in sheep, even though its efficacy is still limited and optimal freezing protocols still need to be developed.

Cryopreservation of ovarian tissue in endangered species

Mammalian ovarian tissue contains a huge pool of follicle enclosed oocytes providing a rich potential source of genetic material. It is therefore of potential great benefit to cryopreserve the genetic material to serve as an oocyte recruitment pool. The best technique to cryopreserve the tissue still needs to be developed. The biological diversity of the ovarian tissue from different species is the most challenging obstacle for the veterinarians and biologists. So far optimized cryo-protocols e.g. for wild cats or for monkeys are currently developed. Cryopreservation of isolated follicles may become a new option, which does not necessitate individualized cryopreservation procedures being developed for each individual species.

Future perspectives

New cryopreservation techniques such as cryopreservation of whole ovaries and isolated follicles using different freezing protocols are currently developed in non human species. These different approaches are essential to develop efficient techniques for the cryopreservation of endangered species to overcome the main obstacle, the biological diversity of ovarian tissues in different species. Coordination of such scientific approaches will be necessary to optimize the efforts and fasten research in the field to efficiently cryopreserve the genetic material of the rapidly growing number of endangered species.

Xenotransplantation and In vitro growth

Xenotransplantation and in vitro growth are techniques, required for the generation of gametes when it is not an option to transplant the tissue into the same species. Several immunodeficient strains of mice and rats may be used as recipients. Furthermore, xenotransplantation of cryopreserved tissue can also be used to evaluate the developmental potential of the tissue prior to transplantation.

Xenotransplantation has been performed with human ovarian tissue as well with tissue from other mammals such as cats and monkeys. Human tissue transplanted under the skin or kidney capsule of mice, and cat tissue under the kidney capsule in rats has been used to demonstrate follicle growth that can be monitored by ultrasound.

In vitro growth is an alternative approach to generate oocytes from cryopreserved tissue. This technique has proven to be successful in mice and sheep as demonstrated by successful births.

However, generation of follicles in vitro still needs further improvement of basic research on follicle physiology and on follicle culture to be used in large mammals.

Future perspectives

Xenotransplantation and in vitro growth are essential techniques if oocytes need to be generated from tissue that can not be transplanted into the same species. Furthermore xenotransplantation is a potential promising technique when there is high risk of metastasis of human cryopreserved tissue that prevents it from being transplanted, as well it may be used as a tool to evaluate the follicular development and quality after cryopreservation procedures.

These techniques are therefore an integral part of the whole research body on cryopreservation of ovarian tissue. The discussions at the workshop between the involved biologist, veterinarians and medical doctors opened new perspectives in the development of integrated strategies to develop this still poorly studied topic.

3. Assessment of the results, contribution to the future direction of the field

Assessment of the results:

It became apparent, that progress in the three fields of ovarian cryopreservation from humans, farm animals and endangered species is different. As shown in Figure 1, in each field several techniques are particularly well established whereas others seemed to be poorly developed.

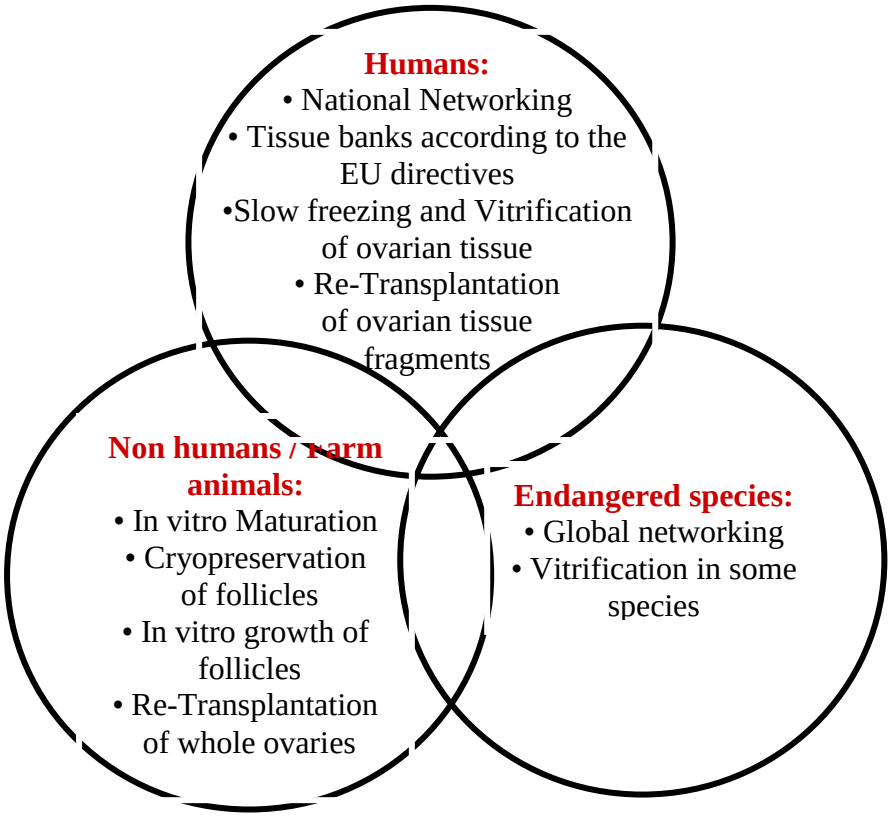
In humans cryopreservation and re-transplantation of ovarian tissue has limited efficacy but has been proven to work as demonstrated by 5 live births. However, essential issues such as networking to provide these techniques to all patients throughout Europe, improvement of efficacy of cryopreservation techniques and the risk of spreading malignant cells, which requires innovative techniques such as follicle cryopreservation, as well as ethical issues urgently need further evaluation in an interdisciplinary context.

In farm animals and other non human tissues such as mice, many techniques are already well developed. Cryopreservation of whole ovaries, in vitro growth of follicles in cryopreserved tissue, isolation and cryopreservation of follicles and xenotransplantation are either already substantially well developed or are currently intensively studied in these species. This field

reveals best scientific progress in the filed of cryopreservation, urgently requiring intensive scientific exchange with the field of cryopreservation in humans and in endangered species.
In endangered species it became clear that global initiatives have already been initiated. However, progress in the development of techniques to store and to use gonadal tissue in order to preserve germ cells is very slow. Some groups around the world and in Europe focus on certain species such as cats and monkeys, exploring some of the available techniques and trying to overcome the differences in the cryobiology of different species. It became obvious that new techniques, as developed in farm animals, could possibly overcome the problem of the species related differences in cryobiology.

Figure 1:

Techniques in the different areas, which are particularly well developed and which can therefore substantially contribute to the development of other areas.



Contribution to the future direction of the field

The workshop has successfully explored the scientific and clinical background, the currently available techniques and the future perspectives concerning the cryopreservation of ovarian tissue in humans, farm animals and endangered species.
The workshop has also revealed that progress in all fields is limited and that this progress could be substantially accelerated by interdisciplinary and multicentre approaches.
As described above, each field provides substantial experience in some techniques which urgently need to be interchanged between the scientists.
The workshop participants came to the conclusion, that an efficient network needs to be established, with the following objectives:

- to create interdisciplinary workshops to discuss future interdisciplinary and multicentre approaches,
- to train and interchange young scientists,
- to establish efficient cryobanks for humans,
- to establish a European initiative to cryopreserve gametes and gonadal tissue from endangered species,
- to address additional topics such as ethics and politics in cryopreservation of human and non-human ovaries.

As these objectives will be ideally met by an ESF networking programme, the participants agreed to head for an application to establish a network starting in year 2010.

4. Final programme

Friday 16 May 2008

08.30-08.45	**Welcome remarks, introduction of all participants, M. von Wolff** (University of Heidelberg, Germany) and **C.Y. Andersen** (University Hospital of Copenhagen, Denmark)
08.45-09.00	**Presentation of the European Science Foundation (ESF)** **Vladimir Bencko** (ESF Standing Committee for the European Medical Research Councils – EMRC) and **Hefin Jones** (ESF Standing Committee for Life, Earth and Environmental Sciences - LESC)
09.00-09.30	**Overview and Discussion of the programme and of the goals of the Workshop**, **M. von Wolff** (University of Heidelberg, Germany)
09.30-11.00	**Session 1: Indication and logistics in Europe in ovarian tissue banking** *Chair*: **M. von Wolff** (University of Heidelberg, Germany)
09.30-10.00	**Tissue banking – humans**, **M. Montag** (University of Bonn, Germany)
10.00-10.30	**Tissue banking – non humans**, **R. Santos** (Utrecht University, Netherlands)
10.30-11.00	**Discussion**
11.00-11.30	*Coffee break*
11.30-13.30	**Session 2: Cryobiology of ovarian tissue and freezing techniques** *Chair*: **C.Y. Andersen** (University Hospital of Copenhagen, Denmark)
11.30-12.00	**Cryobiology of ovarian tissue**, **O. Hovatta** (Karolinska University, Sweden)
12.00-12.30	**Methods of cryopreservation – Slow freezing of ovarian tissue**, **V. Keros** (Karolinska University, Sweden)
12.30-13.00	**Methods of cryopreservation - Vitrification of ovarian tissue**, **J. Lornage** (Hôpital Edouard Henri, Lyon, France)
13.00-13.30	**Discussion**
13.30-15.00	*Lunch*
15.00-18.00	**Session 3: Cryopreservation and transplantation of human tissue** *Chair*: **J. Donnez** (Université Catholique de Louvain, Belgium)
15.00-15.30	**Cryopreservation of whole ovaries**, **B. Martinez-Madrid** (Universidad Complutense de Madrid, Spain)
15.30-16.00	**Orthotopic transplantation**, **J. Donnez** (Université Catholique de Louvain, Belgium)
16.00-16.30	**Heterotopic transplantation**, **M. Sonmezer** (Ankara University School of Medicine, Turkey)
16.30-17.00	*Coffee break*

17.00-17.30	**IVF after transplantation of ovarian tissue**, **C. Y. Andersen** (University Hospital of Copenhagen, Denmark)
17.30-18.00	**Discussion**

Saturday 17 May 2008

08.00-10.30	**Session 4: Cryopreservation and transplantation of non-human ovarian tissue** *Chair*: **R. Santos** (Utrecht University, Netherlands)
08.00-08.30	**Autologous transplantation of whole ovine ovaries**, **M. Imhof** (Medical University Vienna, Austria)
08.30-09.00	**Autologous transplantation of whole sheep ovaries**, **B. Salle** (Centre Hospitalier Universitaire, Lyon, France)
09.00-09.30	**Cryopreservation of isolated sheep primordial follicles**, **C. Amorim** (Université Catholique de Louvain, Belgium)
09.30-10.00	**Discussion**
10.00-10.30	**Discussion within 2 separate subgroups of exerts on human tissue and experts on non-human tissue about future manuscripts and future activities.**
10.30-11.00	*Coffee break*
11.00-13.00	**Session 5: Xenotransplantation and In vitro growth** *Chair*: **V. v. Schönfeldt** (KIDZ Chiemsee, Germany)
11.00-11.30	**Xenotransplantation of human tissue in SCID mice**, **T. Maltaris** (Universitätsfrauenklinik Mainz, Germany)
11.30-12.00	**Xenotransplantation of cat tissue in SCID mice**, **M. Fassbender** (Leibniz Institute for Zoo- and Wildlife Research, Berlin, Germany)
12.00-12.30	**Optimisation of cryopreservation techniques using the monkey model**, **V. v. Schönfeldt** (KIDZ Chiemsee, Germany)
12.30-13.00	**In vitro growth of follicles after cryopreservation of non-human tissue**, **S. Cecconi** (Università degli Studi dell'Aquila, Italy)
13.00-14.30	**Discussion, Discussion of Follow-up activities**
14.30	*End of the meeting*

5. Stastistical information on participants

Gender (n)

- Male: 8
- Female: 9

Country of origin (n)

- Austria 1
- Belgium 3
- Denmark 1
- France 2
- Germany 5
- Italy 1
- Netherlands 1
- Spain 1
- Sweden 2
- Turkey 1

Professions (n)

- Biologists: 6
- Medical doctors: 7
- Veterinarians: 4

The above numbers include the 2 ESF Representatives.

6. List of participants (n=19)

All invited participants did attend the meeting.

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