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Additional Information

## **TITULO: Human Biomonitoring Study of food contaminants, focused on Spanish children: design and lessons learned**

Human Biomonitoring studies, based on measuring the concentrations of chemical substances or their metabolites in human biological matrices, are highly useful for evaluating individual exposure to environmental contaminants and are being carried out in increasing numbers all over the world. The aim of the present work was to describe the methodological framework for transversal Human Biomonitoring studies carried out in the Autonomous Community of Valencia (Spain), analyse the experience and provide the lessons learnt from its implementation in the Regional Health Department's first HBM Program to evaluate the child population's (6-11 year olds) risk and exposure to food contaminants (BIOVAL).

### **1. Introduction**

Health can be jeopardized by exposure to a wide variety of chemicals through different routes, such as inhalation, ingestion, and dermal absorption. Traditionally, the risk due to this kind of hazard has been assessed determining the concentration of pollutants in a specific environmental medium. The main drawback of this method is that an accurate result for human exposure via multiple pathways requires both quantitation of pollutant levels in multiple means and data on individual behavioural patterns affecting exposure, such as the consumption of contaminated foods. In addition, these determinations do not provide data about real quantities absorbed by the human body (body burden), which depend on different factors such as the physicochemical properties of pollutants and their concentration in a specific environmental medium, time of exposure, as well as individual factors such as metabolism or excretion ratio characteristics (WHO, 2015).

In the last decade a new metric, known as Human Biomonitoring (HBM) has been introduced to determine the impact of chemicals on health. HBM is commonly understood as a method for assessing human exposure to natural and synthetic compounds. This new proposal is based on the analysis of biomarkers (BM), which are defined as chemical substances or their metabolites measured in human biological matrices such as blood, urine, hair, adipose tissue, teeth, saliva, breast milk or nails (CDC 2005, EFSA, 2015). The great advantage of HBM is that it permits an integrated measurement of the amount of a contaminant absorbed by the human body, through the different routes of exposure, from all sources of contamination and takes into account inter-individual variability such as metabolism, ratios of excretion and lifestyle (Angerer et al 2007; Joas R et al 2012). In addition, this type of cross-sectional study provides a snapshot of the health and exposure of a population at a specific time (Kleinbaum et al, 1982; Knudsen et al 2012; WHO 1983).

In 2003, the European Commission adopted the "European Strategy for Environment and Health" (SCALE Initiative), whose ultimate objectives were: to reduce the disease burden caused by environmental factors, identify and prevent new health threats caused by environmental factors and to favor the development of health policies in the EU. One of the main goals of this strategy is to protect children against adverse health effects due to their vulnerability to environmental risks (UE-COM 2003). The SCALE Initiative was followed by the European Action Plan on Environment and Health from 2004 to 2010, where the need to develop a good database to collect information was highlighted. The commission underlined the need to apply a coordinated approach to human biomonitoring (HBM) in order to increase the effectiveness of environmental impact assessment, including the effect of food. (European Environmental and Health Action Plan 2004-2010). In this framework, the EU promoted a human biomonitoring program at European level in which 17 European countries (including Spain) participated in order

to create a HBM framework, harmonizing HB procedures and creating a common baseline of results (Joas et al., 2012).

Following this initiative, the Health Department of the Valencian region (Spain) carried out a cross-sectorial HBM study (BIOVAL), whose main objective was to determine the exposure and risk assessment of children (from 6 to 11 years old) due to the presence of contaminants in food in this region. The aim of the present work was to describe the methodology developed for this HBM study, discuss the results and the lessons learned, and make recommendations for new studies.

## **2. Methodology**

### **2.1. Objective**

The design of any scientific study depends on its objectives and the underlying hypotheses (Becker et al, 2014). With this aim in mind, researchers need to define the target population and the pollutants under the study.

The aims set out in the BIOVAL program were similar to those carried out by other HBM studies such as the Canadian Health Measures Survey (Haines DA and Murray J., 2012) or NHANES USA (Calafat, Antonia M., 2012). Specifically, the objectives were: i) to establish reference values (RV) for the different biomarkers of exposure to the main food contaminants, focusing on the population of the Valencian region (ii) to determine the exposure to the selected substances for the general population, (iii) to evaluate the temporal evolution of the biomarkers of exposure and analyse the geographical differences in relation to the exposure (iv) to provide internationally comparable data; (v) to offer data for risk assessment; (vi) to evaluate the effectiveness of different programs, which were focused on the reduction of exposure to pollutants and (vii) to provide recommendations for risk reduction.

#### *2.1.1. Target population*

HBM studies have been used for more than 100 years in occupational health as part of a preventive strategy in the medical surveillance of workers. However, currently, they are recognised as an appropriate means for detection of risks or control of trends in other fields such as public health, environmental health research, public health surveillance and awareness raising (Casteleyn et al. 2009).

In order to define the target population of a study, the geographical location, age, sex and if appropriate the occupation of the subjects, as well as the criteria of inclusion (characteristics that make a unit part of a population) and exclusion criteria (characteristics whose presence makes a unit not part of a population) must be determined precisely. The target populations selected in the different HBM studies carried out in recent years has been very varied. Taking as reference a review made by the University of Copenhagen (EFSA 2015) based on thirty-seven HBM studies carried out in different countries in recent years, different examples can be observed: for example the Flemish Environment and Health Study in Flanders (new-borns and their mothers, adolescents aged 14-15 and adults aged 50-65 years old); the Canadian Health Measures Survey population (from 3 to 79 years); and the National Health and Nutrition Examination Survey (NANHES) in the United States, (population over 2 months old).

On the other hand, the European Strategy on Health and Environment (SCALE initiative), and the Declaration of Parma (WHO 2010), emphasise protection of the health of children, who are especially vulnerable to environmental risks.

Therefore with the aim of being able to compare our results to those obtained in other HBM studies (CDC, 2009, Health Canada, 2009, COPHES / DEMOCOPHES, 2010- 2012), the target population selected in BIOVAL was the population from 6 to 11 years old in the Valencian region (Spain) without any metabolic disease.

### *2.1.2. Pollutants*

The reason why a pollutant is chosen should be defined previously. In BIOVAL, as it is a study carried out by the Department of Health in the framework of the actions in Food Security, the contaminants selected had food as a main route of access to the human body. The selection was based on both European and Spanish food safety legislation (AECOSAN). The results from the evaluation of exposure to food contaminants at European and international level, recommendations derived from the Consortium to Perform Human Biomonitoring on a European Scale (COPHES) as well as HBM programs developed in other countries (GerES, CDC - National Biomonitoring Programs) were also considered. The persistence and bioaccumulation of the substances and their toxicological characteristics were also taken into account. The result of this selection is shown in Table 1.

## **2.2. Study Design**

### *2.2.1. Matrix and Biomarkers*

Regarding matrix selection different authors indicate as main characteristics of the ideal matrix for biomonitoring studies: it should be accessible in sufficient amounts without a risk for the donor, it should reflect the body burden, it should contain detectable levels of the biomarker in terms of the analytical methods available, and it should be easy to collect (non-invasive) and store (Esteban and Castaño, 2009).

Blood was the most commonly selected matrix (67% of the studies), followed by urine (57% of the studies) and hair (35% of studies) (EFSA 2015). The main advantage of blood being in contact with different organs and tissues and is in steady state with all of them (Angerer J. et al 2007). Depending on the type of biomarker, measurements can be performed in whole blood serum, plasma, or specific cell types (e.g., lymphocytes). The invasive nature of blood sampling has some important drawbacks: it negatively affects the participation rate of study participants, the amount of sample is often limited, and sampling in young children or infants has practical and ethical downsides (Alves, A. 2014).

Another important matrix is urine. It is a non-invasive matrix, which is accessible in a large volume, allowing the determination of very low concentrations of chemicals caused by environmental exposure, particularly of hydrophilic metabolites, such as those from currently-used organophosphates, pyrethroid insecticides or different classes of herbicides (Barr, 2008). Non-persistent chemicals are quickly transformed to hydrophilic, polar metabolites and excreted mainly in urine (Koch and Calafat 2009). The main advantage is that spot samples are easy to collect, however, the varying volume and the consequent dilution of the target compounds are the major disadvantages (Alves, A. 2014) In order to avoid this inconvenient, the two most commonly solutions used are: expressing the results per gram of creatinine and adjusting the measured values for the specific gravity of the measured compounds. Since, the amount of

creatinine excreted indicates how strongly the urine is diluted. The value is used to classify the relative exposure to other substances (GerEs V). Many studies have focused on exposures in young children, particularly organophosphorus insecticides and pyrethroids exposures (Muñoz Quezada et al 2012, Quiros Alcala et al, 2011, Bouchard et al, 2011, Shalat et al., 2003, Becker K et al, 2006).

Hair and nails are also non-invasive matrix, easy to collect and that can provide information about short-term to long-term exposure (months or even years), which is not always possible for blood, plasma, or urine analysis (Cooper G et al, 2012). But this matrices have disadvantages as lack of sensitivity for several compounds, potential contamination through the use of medication, nail polish, external deposition of chemicals and also have limitations related to the interpretation of toxic-kinetic.

In order to choose the biomarkers and biological matrices correctly, both the velocity and the metabolic pathways as well as the types of metabolites and their excretion concentrations through the different routes of metabolization must be considered. In addition, the availability of validated analytical techniques with detection limits adjusted to the values of exposure biomarkers we expect to find should also be considered (WHO 2015).

In BIOVAL, two matrices were considered: first-spot morning urine and hair were the. Both of them permit an easy obtaining of the matrix (since the target population were children) and was also considered the routes and rates of the pollutants metabolization and the reference values developed (Table 1).

### *2.2.2. Sample population*

In relation to the ideal size sample, there is little bibliographical references about it, although it is true that the recommendation of the International Federation of Clinical Chemist (IFCC) consider that in cross-sectorial surveys is recommended a minimum of 120 randomly selected individuals per population group to allow for the determination of 95th reference percentiles and their 95% confidence intervals (Poulsen et al, 1997) and other HB studies by different authors are also consistent with this sample size (Castaño et al.,2012; Becker K. et al, 2014 Esteban M et al, 2015 , Berman et al.,2011, Volkel et al., 2008; Kubwabo et al.,2004). Therefore, the sample size defined in BIOVAL, was determined following the criterion of the 95th percentile, was also taken into account the distribution of children in the three provinces that make up the Valencian Community (INE, 2015), analytical capacity of our laboratories and the existing Public Health Centers in Valencian Region (CSP). Resulting in a sample size of 630 participants, whose distribution by provinces is shown in table 2.

This distribution was made establishing a recruitment of 30 children per school, being 35 children per school the maximum limit. Our study was carried out during 2016. The activities carried out were: the design of the standard operational procedures of the study (SOPs) and its implementation, which also included the managers training to conduct the field work properly, the recruitment of participants, to carry out the surveys and the collection of biological samples (hair and urine).

## **2.3. Field work**

In order to ensure the quality of the data obtained in the different field activities it is essential that these be carried out in a standardised form, and we therefore compiled a field work manual as had been done in similar HBM studies (COPHES/DEMOCOPHES). This manual included an agenda for all the activities to be carried out, plus detailed instructions on how they were to be conducted, and was uploaded to a web page to which all the staff at the Public Health Centres (PHC) had access.

Also, as we were aware of the vital importance of the training of those involved in the field work for the success of the project, a training course was organised for all 42 individuals responsible for conducting this stage in each of the 16 PHCs.

### *2.3.1. Recruitment process*

The first stage of the field work is to recruit volunteers that satisfy the defined inclusion criteria from the selected universe. Voluntary participation is a legal requirement in this type of study. The volunteers can be recruited from a number of sources, according to the objective and the defined universe of the study. If children are to be used, recruiting can be carried out in kindergartens or schools; newly born children or those affected by specific pathologies can be recruited from hospitals; and participants from the general population can be recruited from records such as census lists, etc.

The first contact with potential subjects can be made in a number of ways; many authors favour telephone calls (Hertz-Picciotto, et al, 2010), while others propose direct contact at the school gate or by newsletters. (Fiddicke U, et al, 2015), or visits to prenatal clinics to directly invite possible subjects to participate, as in the National HB program in Slovenia, (Perharic L et al, 2012).

Another decision that must be taken regards whether or not the participants are to be given an incentive. Many HBM studies have considered this appropriate due to the inconvenience caused to the subjects (Voigt M. and Eis,D., 2004; Arcury T et al, 2010; Cърna M, et al 2012; Becker et al, 2014).

With the above cases as reference, and bearing in mind that the present study aimed to determine the exposure of primary school children (6-11 years of age) to food contaminants, it was decided to carry out the recruitment in schools. For this we had the participation of 16 PHCs that selected the representative schools to be contacted. No consideration was given to rewarding the participants in any way.

Figure 2 shows the stages followed by the BIOVAL program in the recruitment process. It should be pointed out that in order to encourage participation, those in charge of recruitment followed a flexible policy as regards dates and contact times with the children's parents.

### *2.3.2. Informed Consent & Questionnaires*

The consent form includes all the information provided to possible subjects and which must be signed by the volunteers or their parents/guardians in the case of minors, as in the present study. The signing of this document is an essential condition for a person to be considered as a possible participant. In this case, all the participants signed an *informed consent form* (see Supplementary Information), which included information on the Biobank for Biomedical Research in Public Health of the Community of Valencia (IBSP-CV), which is part of the

Valencia Biobank Network and the National Biobank Platform. Also included was a request to consent to the storage of any excess of biological samples.

As regards the treatment of the data obtained in the study, this should be in accordance with the current national and international standards on data protection and participants should be informed of the use to be made of it. In BIOVAL, the standards of the Spanish code (S.N. 1999 a, S.N. 1999 b) were adhered to. The participants were informed that the information would be published only in scientific journals and would not include any personal details. The program was approved by the Ethical Committee of the Department of Public Health and the Centre for Research in Public Health.

The questionnaires were carefully designed as the information they contain is fundamental for the results obtained; the methods chosen must be carefully considered and in many cases it is advisable to follow the standard design used in previously published studies (WHO, 2015). After a review of the literature (*Encuesta de Nutrición de la Comunidad Valenciana, 2010, INMA Proyecto...*) it was decided to divide the questionnaire into three sections: a) social-demographic information of the participants, parents' occupation, type of home; b) 72-hour reminders and c) the frequency of consumption by food groups during the previous week. The questionnaires can be seen in *Supplementary Information*.

The questionnaire was validated by 12 participants. The objective of this stage was to observe individual reactions to each question in order to confirm the clarity and ease of comprehension of the questions and instructions, obtain an exhaustive identification of all the response options and to estimate the average time necessary to answer all the questions (Downing, S.M., 2006, Downing S.M. & Haladyna, T.M. 2006, Schmeiser, C.B. & Welch, C., 2006, Wilson, M., 2005). The number of participants in the survey pre-text was defined in accordance with the general recommendation to use small groups of between 12 and 30 individuals (Anderson, J.C., & Gerbin, D.W. 1991; Hunt, S.D., et al, 1982).

After approving the definitive questionnaire, in order to obtain the maximum participation it was decided to offer the parents two options: fill in the questionnaire in the school aided by the PHC staff or to do so at home with a contact telephone number in case of any doubts or questions they might have.

### 2.3.3. *Biological samples: collection, transport & storage*

The collection, transport and storage of biological samples should be done in accordance with the existing guides and recommendations for the selected matrix study and the biomarkers to be analysed.

In BIOVAL the matrices selected for biomarker analysis were the first morning urine specimen and a hair sample. The sampling, transport and storage procedures for the urine specimens followed the international guides and the Expert Team to Support Biomonitoring in Europe Protocol for sample collection (ESBIO, 2004). For hair samples, the recommendations of the *Instituto Carlos III de España* were followed. Details of both these guides can be found in *Supplementary Information*.

Each participant was issued with a kit containing disposable gloves, a sterile 100 ml polypropylene bottle and instructions on how to take urine samples at home first thing in the morning. Each morning the participants delivered the sample to a team from the Health Centre at their school. The samples were kept at 4°C in a portable refrigerator and within two hours were

taken to the Biobank, where they were divided into aliquots and stored at -80°C until analysis, following the normal procedures. PHC staff members obtained hair samples at the schools. These were then stored at ambient temperature and protected against damp while being transported to the Biobank.

## **2.4. Sample Analysis**

The selected analytical methods should be sufficiently specific and sensitive and within the proper quantification limits for the level expected in the population under study. The expected concentration of biomarker in the selected matrix will thus be taken into account, as well as the complex composition of the biological matrices that make it necessary to apply clean-up and pre-concentration of analytes to eliminate interference from other chemical products and enrich the target analyte to detectable levels (WHO 2015).

In the second phase, the analyses of the biomarkers were carried out in the Public Health Laboratory of Valencia and Public Health Laboratory of Alicante (Spain). Table 1 lists the biomarkers analysed and the previously validated techniques used.

## **2.5. Data analysis**

Data analysis is carried out with the support of statistical tools. Published studies describe the use of different treatment techniques according to the required objective (Becker et al. (2006, WHO, 2015).

BIOVAL included: i) a descriptive statistical treatment to establish criteria for treatment of infrequent data and with results with values below the quantification limit, ii) a study of exposure predictors using multivariate analysis tools, and iii) the risk evaluation for those biomarkers that currently have health-based guide values (HBMI, HBM II and BE)

## **2.6 Communicating the results**

The strategy for communicating the results should be included in the project design and thus should appear in the events calendar. This strategy is defined according to the objectives of the study and the groups for which it is intended. A review carried out by the University of Copenhagen on different HBM studies in Europe (EFSA 2015) describes the different results communication strategies employed, the most common of which is the use of reports and communications to the participants (Becker et al, 200, Vrijens, J. et al, 2014, Pérez Gomez et al, 2013).

In the present study two methods of communication were used: one directly to the volunteers through their school and another to the general population through scientific journals and possible participation in congresses and seminars.

## **3. Results and discussion**



The study in general was carried out successively and encountered no problems, which confirmed the viability of the chosen strategy. However, it also showed us certain limitations and aspects that could be improved in future biomonitoring studies.

### **3.1. Planning and training**

In order to ensure the use of standardised methods in BIOVAL, a field work manual was compiled containing the procedures detailed in Table 3 (see also *Supplementary Information*). A training course was also organised for the 42 members of staff at the 16 Public Health Centres who were involved in the field work; this included a description of the general program and specific tasks. At the end of the course they were given: i) information in digital form for the introductory meeting with parents, ii) a field work manual, and iii) instructions for taking biological samples.

The field work manual and the training given, in line with the European DEMOCOPHES (Fiddicke U., 2015), made it possible for the different field work stages to be carried out consistently by the different teams and to ensure the quality of the results. At the end of the training course the participants filled in a questionnaire (see responses in Figure 3), which showed a high degree of general satisfaction.

### **3.2. Field-work**

After completion of the field work, the supervisors of the PHCs filled in a survey designed to identify the difficulties involved in each stage, specifying the most important problems found in each one and suggesting ways in which they could be improved. This allowed us to identify efficient methods and learn lessons for future editions of the BIOVAL program.

#### *3.2.1. Recruitment process and lessons learnt*

Conscious of the importance of the commitment and attitude of the school management on the scheme's success, the members of the PHCs were able to select appropriate schools based on their interest on participating and discard those that showed a negative interest. This meant that the work teams had the schools' active support and also encouraged parents to take part, which greatly facilitated the teams' work.

In order to obtain the volunteers' commitment to the study, it is essential that they have an interest in its aims, and this means making them see that the results can help them improve their habits; otherwise it is preferable for them not to take part as they could provide incomplete information or give what they think is the required answer. They must therefore be put under no pressure at all and be made to feel at home in an atmosphere of trust, transparency and professionalism. Another important point is that they should be aware beforehand how much time they will need to dedicate to the tests; our experience of this aspect is in agreement with the work done by (Hertz- Picciotto et al, 2010), in which the lack of time is the most frequent reason given for not participating. It is therefore important to come up with an accurate estimation of the time involved and duly inform them about it. Experience has shown us that if the volunteers see that the tests need more time than they had been told, they will tend to leave questions unanswered or give incomplete information. Table 4 gives the results obtained in relation to those expected. It can be seen that the final number of participants was 666, which was over the programmed minimum number of 630, of which 51.5% (343) were boys and 48.5% (323) girls; 51.5% were between 6-8 years old and 48.5% (323) between 9 and 11.

These results show that the strategy followed was appropriate, however the authors would like to point out that although the attitude of all the schools involved was positive, there were large differences in the numbers recruited by the different teams. While some obtained more than the expected number of volunteers with ease in a single campaign, four of the sixteen teams had to resort to an additional school to obtain the minimum number of volunteers, due to the low numbers of parents who attended the preliminary information sessions.

The team supervisors were asked to complete a survey on the difficulty involved in this stage. The results showed that 81% reported no difficulties, while 19% found minor problems which mostly involved: parents' lack of available time (30% of those surveyed), non-attendance of parents at the information sessions (25%), low educational qualifications of the parents (13%) and the remainder were either "don't know" or "can't say".

The structure, presentation and questions in the questionnaire are essential to obtaining the most reliable information (Becker, k. et al, 2014) and the use of the Zimbabwe method of presentation was found to be a fast and easy way of calculating rations in complying with the 72-hour reminders.

As regards the options in filling in the surveys, Table 5 shows the different methods and lists their advantages and disadvantages. In the present study the participants were given two options: face-to-face interviews or completing the form themselves. The first option was mostly rejected and only 2.3% of the parents opted for it, citing a lack of time. However, in the second case we detected several cases of inconsistency, misinterpretation and unanswered questions, which made it necessary to contact the participants for clarification. However, in many of these cases we had great difficulty in locating the parents and in some cases it was impossible. The authors therefore consider that more reliable information is acquired in the face-to-face interview.

The BIOVAL program made use of the CAPI application (**QUALTRICS**), in which each PHC entered the results of the questionnaires completed by the participants in their zone.

This stage was assessed as being the most difficult by the field work team leaders; 38% found it to be of average difficulty, 31% quite difficult, 25% low difficulty and 6% had major difficulties. The main problems encountered were: 48% found the questionnaire too long and/or too complex, 28% found the food items difficult to interpret, 16% had to deal with parents with little interest in cooperating and 8% with parents who could not remember the food items consumed by their children.

**The lesson learned from the experience** was that the questionnaire was in general too long, especially the part regarding food items and some parents found them difficult to interpret, while some had little interest in cooperating. The authors are of the opinion that future questionnaires should be as short and simple as possible and provide the participants with clear information based on a rapid evaluation of an aspect of the participants' diet. This would have two advantages: it would increase the participants' interest, and thus improve recruitment and the standard of the responses, since in answer to their questions the participants would obtain rapid feedback on an aspect of their children's diet.

### 3.2.2. *Sampling process*

Other Human Biomonitoring studies used different strategies to collect biological samples; e.g. in a Canadian HB study of environmental chemicals (Douglas et al, 2012) a mobile examination centre was used to examine and take samples from the participants. In

DEMOCOPHES- España (Esteban López, M et al, 2015) the samples were collected either in the participants' homes or in special centres set up for the purpose.

In the present study, the participants gave urine samples in their own homes, for which they were provided with a pack containing the necessary materials and instructions. Hair samples were collected in the schools by PHC staff and no incidents or problems were reported. The results are given in Table 4; it can be seen that 100% of the participants provided a urine specimen, of which 3.9% were below the quantity required (100 ml). Of the 666 participants, only 3 (0.45%) refused to give a hair sample.

94% of the field work team leaders reported little or no difficulty involved in this stage, while 36% had problems with insufficiently long hair samples, 21% had to deal with reluctant children and 7% with the organisation.

**The lesson learned from this stage** was that as the scheduling of collecting samples and surveys was carried out at the school gates, even though this was done in several stages, having to deal with large numbers of participants and the fact that the parents were usually in a hurry to get to work indicate that in future studies this work should be done by a larger number of PHC staff.

#### **4. Conclusions**

BIOVAL is a pioneering HMB program designed to assess the internal exposure to food contaminants in the child population of the Community of Valencia. Its development and final implementation also allow us to evaluate risks for biomarkers that currently have health-based values (HBMI, HBM II and BE). The results of this first stage have enabled us to determine the viability of the strategy and have identified aspects that could be improved in future biomonitoring studies. One of the main problems in recruiting is the shortage of time available to families with small children, especially when both parents work, so that it is essential to adapt HBM programs to their needs in order to reduce the time required to the minimum. We also consider it advisable to be flexible in collecting the responses to the surveys (face-to-face, telephone interview, etc) in order to adapt to the parents' circumstances, facilitate recruitment and avoid losing participants in the course of the study.

The quality of the results is completely dependent on the collaboration of the volunteers. Any schools or parents not totally committed to collaborate and/or with no interest in the results should be excluded. Also, those in charge of the study should make collaboration as easy as possible by means of a questionnaire that provides immediate feedback information to the participants. Finally, availability and transparency should be the pillars on which the trust and interest of the volunteers are founded.

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